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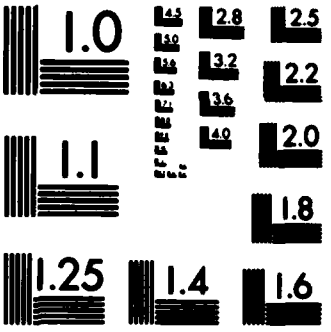

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This report describes further progress in characterizing detergent soluble DNA from activated murine splenocytes and lymphocyte cell lines. It also describes experiments implicating the involvement of DNA replication in detergent soluble DNA synthesis and disproves the assumption that detergent soluble DNA is a random degradation process.

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SUMMARY OF RESEARCH PROGRESS
June 1983-October 1983

Phyllis R. Strauss
021-30-7461

The purpose of this contract is to charactize cytoplasm-associated DNA from murine spenocytes and to determine its physiological function. Throughout the report, I will refer to this DNA as "MADS" DNA, metabolically active detergent soluble DNA.

I. Characterization

A. The manuscript entitled "Characterization of rapidly-labeled detergent soluble DNA in murine lymphocytes" has been accepted for publication by Biochemistry (Washington). A copy is enclosed as Appendix I.

B. The question of whether each size class in MADS DNA is a unique sequence or contains common sequences with other size classes has moved more slowly than anticipated. Our initial attempts to clone the material 'shotgun' (that is, try it and see what happens) were not successful. Therefore, we needed a restriction analysis before proceeding. When Dr. Harry Meade (Biogen Corp, Cambridge, MA) was not forthcoming with a restriction map, we decided to invest in the required restriction endonucleases. The experiment was designed to maximize sensitivity and minimize the amount of MADS DNA needed by labeling either whole MADS DNA or one of its fragments with ^{32}P-phosphate using the nick translation system. The ^{32}P labeled material was exposed to each of a battery of restriction endonucleases and size changes were observed by autoradiography

after electrophoresis on agarose gels. Although the nick translated starting products of MADS DNA had themselves become smaller than native MADS DNA or its fragment, no difficulty was encountered with ϕx174RF/Hae III control DNA. Despite this problem, nick translated MADS DNA fragments were treated with Eco RI, Acc, HindIII, SmaI, HincII, BglI, SauIIIa, MboI, HpaII, ClaI, HaeIII, PstI, MspI, Kpn, AluI, AvaI, BalI and BclI. Only HaeIII resulted in cleavage. The result with HaeIII was expected as it had been observed earlier and reported in the manuscript appended. However, the lack of effect of the array of other restriction endonucleases was not expected, especially in light of the presence in MADS DNA of a variety of DNA sequences including those of globin β chain and immunoglobulin κ chain. In evaluating these results we decided to investigate three additional restriction endonucleases because these are known to cleave mouse satellite DNA. The three, AvaII, EcoRII, and Sau96I, cleaved MADS DNA, the cleavage pattern with AvaII being similar to that of HaeIII and different from Sau96I and EcoRII. Although we could not draw conclusions about the similarity of MADS DNA and mouse satellite DNA, all the enzymes which cleaved MADS DNA contained GGNCC sequence specificity. Thus, it is significant that we initially reported a slightly higher GC content in MADS DNA than in bulk chromosomal DNA. Now that we have cleavage sites, we can generate blunt ends on MADS DNA fragments, attach linkers and insert the DNA into cloning vehicles.

C. Pulse labeled MADS DNA contains very rapidly labeled sequences smaller than 100 base pairs. These were end labeled at the 5'

terminus with ^{32}P-phosphate using polynucleotide kinase and analyzed on Maxam-Gilbert denaturing gels (Maniatis etal., Molecular Cloning, Cold Spring Harbor Press, 1982). They were a discrete size between 10 and 14 base pairs in length. Since they were so short, it should be possible to sequence them by chemical methods. Sequencing experiments are in progress.

End labeling with polynucleotide kinase did not result in labeling size classes larger than the 8-12 mer. This observation has held even when labeling was performed under conditions for recessed 5' ends (3' overhang) or exchange conditions. We have not yet exhausted all possibilities, however, since before a new 5' phosphate can be added, the original 5' unlabeled phosphate must be removed with either calf or bacterial alkaline phosphatase. The conditions for removing 5' phosphate in the presence of a 3' overhang are far more rigorous than those we have previously employed. Experiments are underway towards this end. If we are still unable to label at the 5' end, it will be necessary to explore alternative reasons such as covalent linkage of other molecules either protein or RNA. Because we have had difficulty in end labeling at the 5' terminus, we end labeled MADS DNA at the 3' terminus using terminal deoxytransferase and cordycepin 5' triphosphate. Although better results were obtained, not all species visualized by ethidium bromide staining patterns were labeled. We consider the possibility that a 3' overhang is folded back onto itself in most MADS species.

D. Electron microscopy is a useful tool in analyzing unusual DNAs because it allows the investigator to examine single pieces for irregularities. We are currently examining MADS DNA and its isolated fragments under native and denaturing conditions. Although quantitative measurements have not yet been made, it is clear that larger fragments in MADS DNA contain bubbles and forks in what appears to be a regular fashion. This would confirm the predictions made in I-B, that MADS DNA contains single stranded regions.

E. Early attempts to determine which lymphocyte class (T or B) contained MADS DNA by means of differential sensitivity to immune sera were unsuccessful because controls treated with complement in the presence of calcium were also depleted of 3H-thymidine labeled MADS DNA. This was not true if calcium was not present in the medium. Divalent cations are notorious requirements for both nucleases and some proteases. The nuclease problem is insoluble because there are no good inhibitors of nucleases other than chelating divalent cations with EDTA or EGTA. The protease problem could be explored directly because there are several good protease inhibitors such as phenylmethylsulfonylflouride (PMSF) available, and amino acids covalently linked to viruses and mitochondrial DNA have been recently reported.

To determined whether MADS DNA contains covalently linked protein, NP40 supernates were prepared in the standard fashion except that PMSF was added as a protease inhibitor. MADS DNA was isolated as usual except that the initial step employing proteinase K was omitted. The final preparation was divided in half and one half was

subjected to treatment with proteinase. Both halves were analyzed by agarose gels under native conditions. The portion which was not treated with proteinase K behaved exactly like the standard preparation. Therefore, we conclude that MADS DNA does not contain a long chain of covalently linked amino acids sufficient to interfere with its mobility on agarose gels.

II. Function

A. In the previous progress report we described a series of pulse chase experiments in which a small fragment incorporated 3H-thymidine rapidly (15min) and could be chased uniformly into larger size classes thereafter. The small fragment has been further characterized (see I-C). We have now shown that two inhibitors of replication, aphidicolin and hydroxyurea, did not inhibit the rapid labeling of the small fragment. (See Figures 1 and 2) Both inhibitors, however, prevented the transfer of radiolabeled thymidine to MADS DNA. Therefore, the transfer process was associated with replication. In order to interpret these results, we have examined what happens to 3H-thymidine which is incorporated into the detergent insoluble fraction and which presumably represents chromatin. No rapidly labeled oligonucleotide was found in the detergent insoluble fraction at any time. (See Figure 3) Moreover, all incorporated ^{3}H-thymidine was associated with DNA of higher molecular weight than that found in MADS DNA. A manuscript describing these findings is in preparation.

B. The presence of MADS DNA in several cell lines is under investigation. Jurkat human lymphoma cells continue to synthesize

and label MADS DNA after they have been exposed to Concanalin A or phorbol myristate acetate plus poke weed mitogen, which are reported to stimulate IL-2 production. While we have not measured IL-2 production ourselves, we have observed that the cells aggregated and were very difficult to handle within 12-24 hrs.

Studies on cell cycle dependence in Jurkat cells are also in progress. We have developed a method to separate Jurkat cells on the basis of size by means of Nycodenz gradients. Nycodenz is a new polymer similar to Ficoll but less toxic. The separation appeared to correlate with stages in the cell cycle as analyzed by cell cycle analysis. Cells which had been labeled with ^{3}H-thymidine prior to separation have been analyzed for the distribtution of incorporated nucleoside into detergent soluble and insoluble fractions. A striking increase in the ratio of MADS DNA to chromosomal DNA (detergent insoluble DNA) is seen about one third of the way down the gradient. These experiments are being repeated now and a manuscript will be in preparation shortly.

Further studies on function require a well defined cell line where stimulators and modulators of replication and transcription are known (See below).

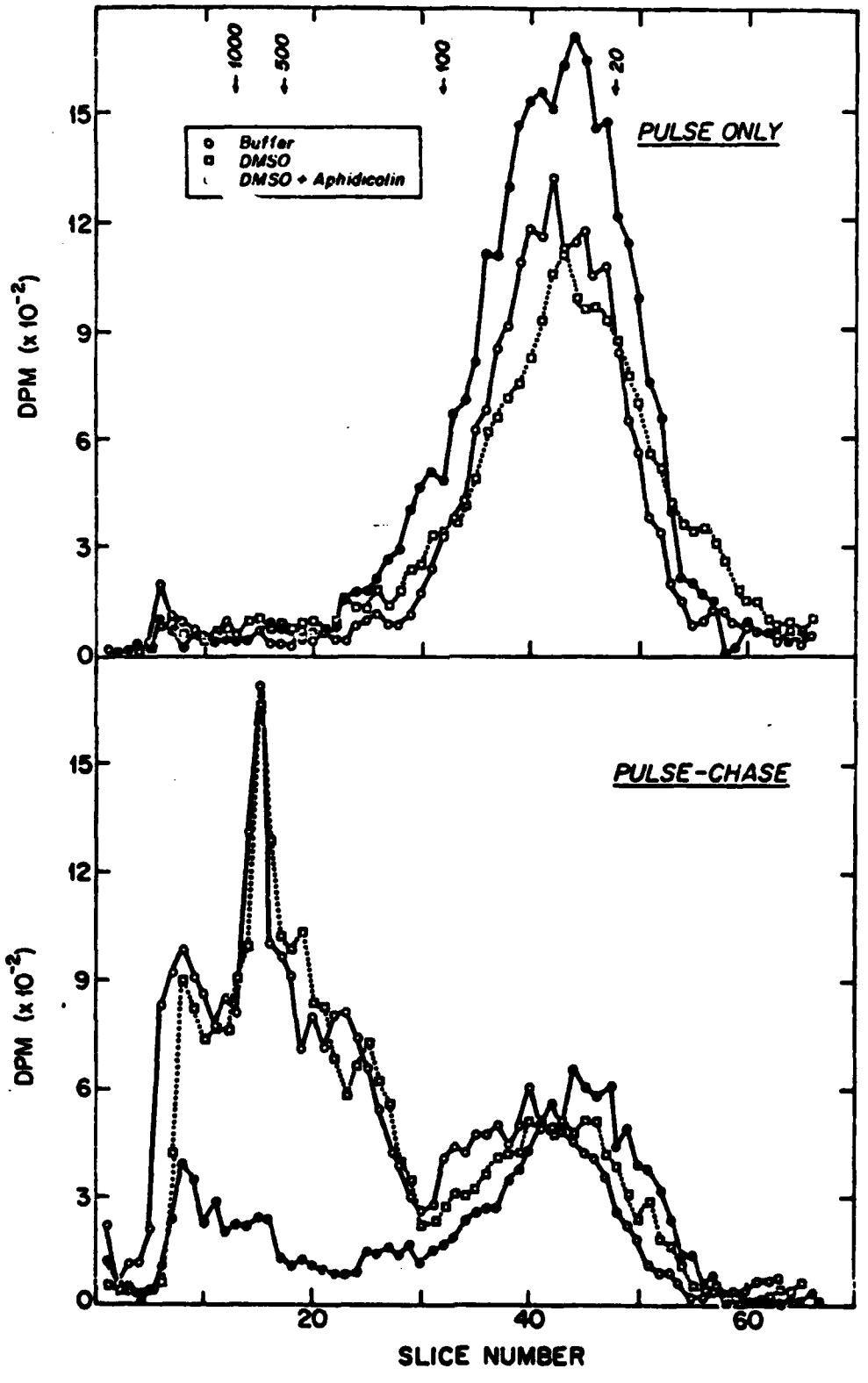

Figure 1: Cells from Con A stimulated mice were incubated in buffer, 0.5% (v/v) DMSO, or DMSO containing 10 μg/ml aphidicolin for 15 min. ^{3}H-TdR was added to 6 μCi/ml. After a 15 min pulse with ^{3}H-TdR the cells were washed and resuspended in medium containing DMSO or DMSO with aphidicolin for a chase period of 60 min. At the indicated time they were washed and detergent lysed. MADS DNA was isolated from the detergent soluble fraction and analyzed by agarose gel electrophoresis (1.2% gel). -7-

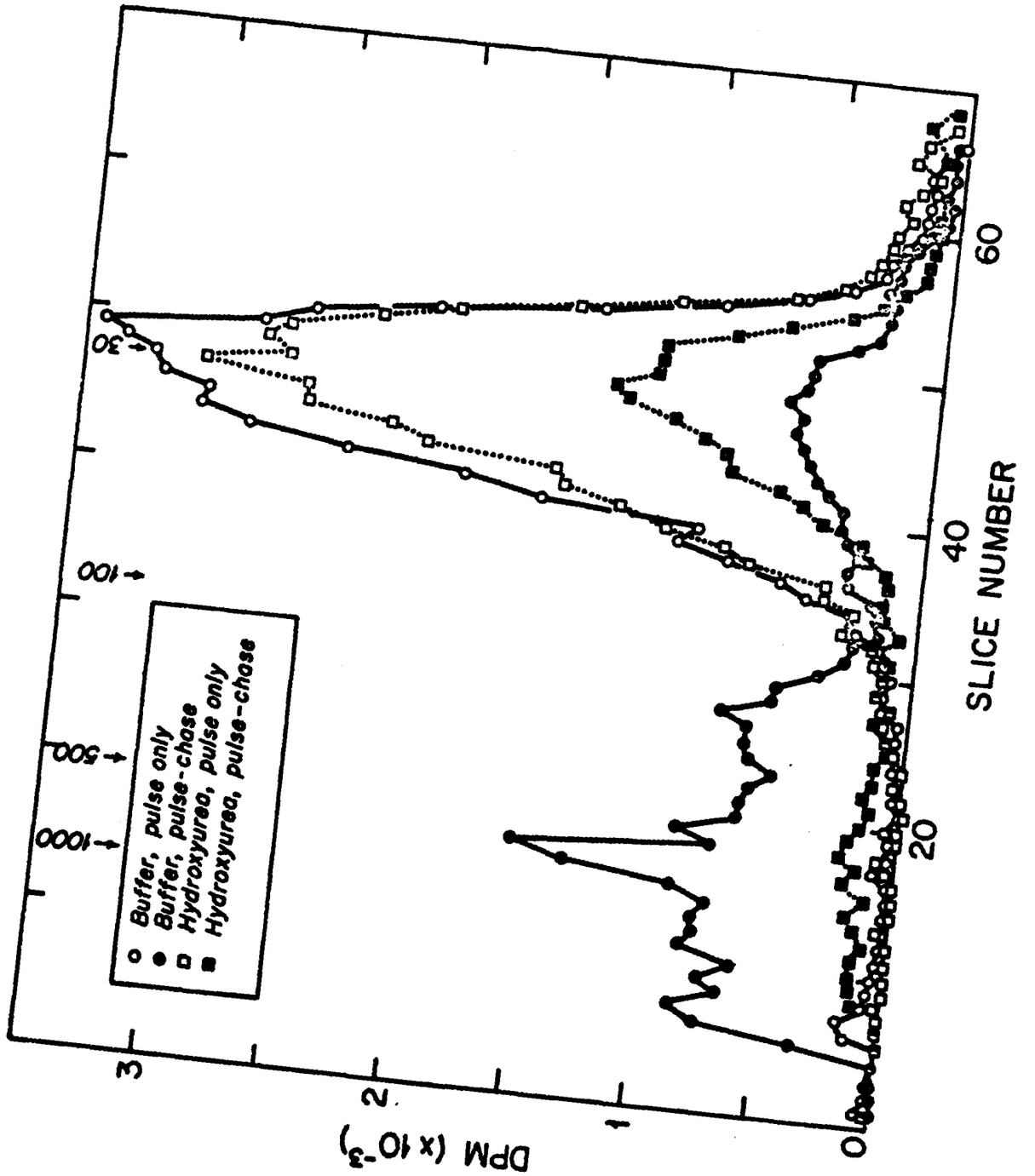

Figure 2: Cells from Con A stimulated mice were incubated in the presence or absence of 2mM hydroxyurea for 30 min at which time ^{3}H-TdR was added to 5 μCi/ml. After a 15 min pulse, cells were washed and resuspended in medium with or without hydroxyurea for a 60 min chase. They were then washed and detergent lysed. HMDS DNA was purified from the detergent soluble fraction and analyzed by gel electrophoresis (1.2% agarose).

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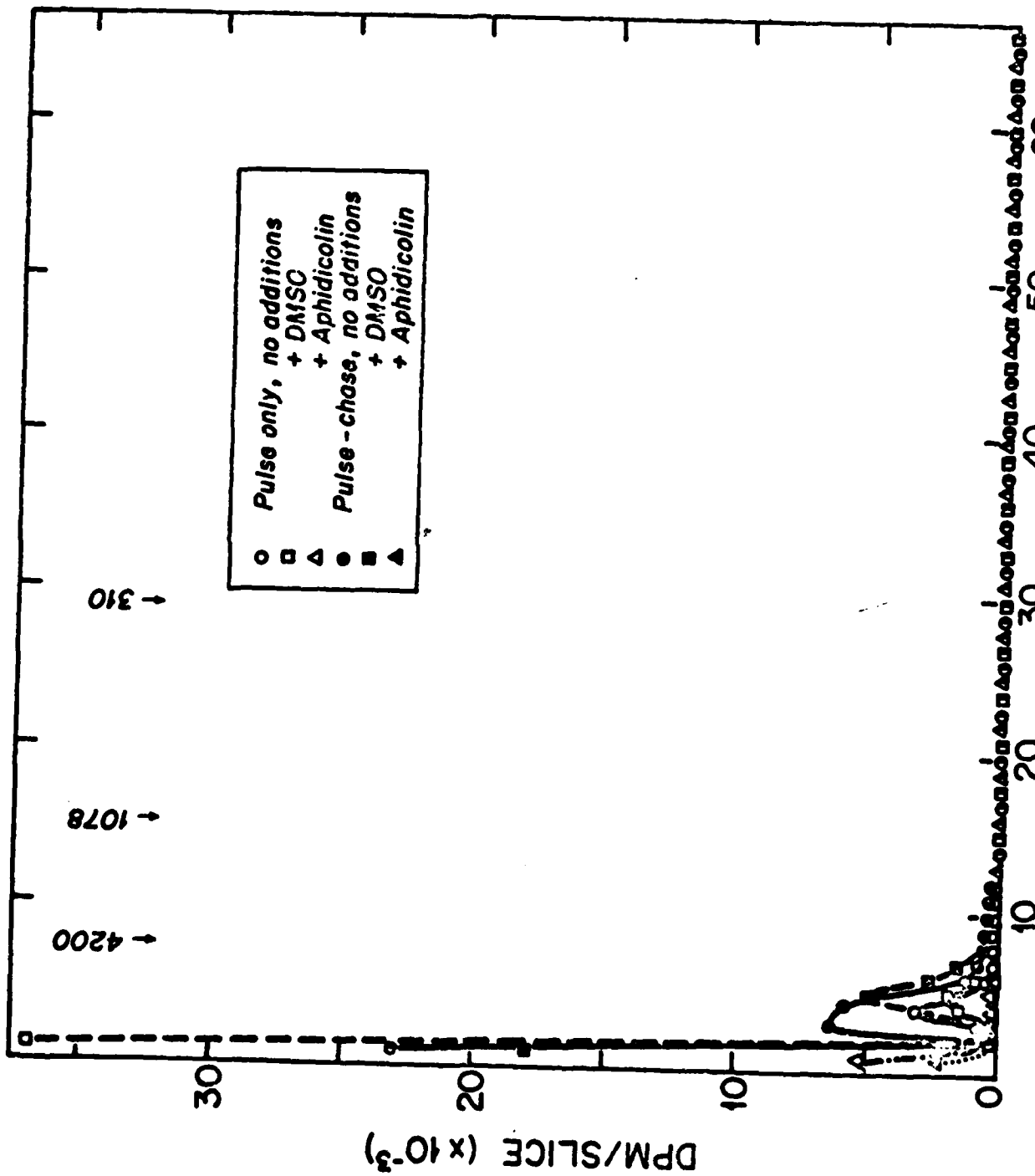

Figure 3: DNA from detergent insoluble pellets from the experiment described in Figure 1 was purified and analyzed by agarose gel electrophoresis.

PROPOSED EXPERIMENTS
March 1984-February 1985

The following experiments are proposed in addition to continuing the work described above.

A. We intend to sequence several purified fragments of MADS DNA from lymphocytes obtained from conA stimulated mice to determine whether there are consensus sequences (rationale described above in IB)

B. Whenever a person versed in the chromatin field sees the MADS DNA results he/she concludes that MADS DNA is nucleosomal in origin on the basis of the size distributions alone. If MADS DNA is nucleosomal, it must represent a special class of nucleosomes since nucleosomes are generally considered to be detergent insoluble. In fact detergent lysis is often used to prepare the nuclei from which nucleosomes are obtained (see discussion in Strauss et al, In press). I propose to centrifuge the NP40 supernate prepared in the presence of PMSF on linear sucrose gradients. The fractions will then be analyzed for MADS DNA and histones. If MADS DNA is part of a nucleosome complex, histones must be present in the correct molar ratios. I also propose to perform a thorough study of the effects of a graded series of detergents on nucleosomes and on "newly synthesized" and "old" chromatin. These studies should answer the question once and for all as to whether MADS DNA is nucleosomal in origin. At this time we have run preliminary sucrose gradients and worked out polyacrylamide gel systems for analyzing histones.

C. The pulse chase studies described in IIA were performed at intervals no shorter than 15 min. In order to study replication intermediates we must reduce the pulse interval to 30 sec. Since a half minute pulse is insufficient to get enough radiolabel into intact cells, we intend to use permeabilized cells as described by Kowalski and Denhardt (Nucleic Acids Res. 5: 4355-4373). The pulsed cells will be followed to determine the fate of radiolabeled materials. We will be sure to collect and analyze the chase supernate because we would expect newly synthesized MADS DNA to be liberated into the supernate.

D. As described in the PROGRESS II-B section, we need to capitalize on a cell line in which replication and transcription are already well studied and can be manipulated. A search for lymphocyte lines has proved fruitless. As noted by the Contract Officer and confirmed by Dr. Emil Unanue (Harvard Medical School Department of Pathology), lymphocyte lines are already committed to an immunological function or unable to be committed at all. The lack of a manipulatable line has seriously hampered the understanding of the immune process, according to Dr. Unanue. Friend's erythroleukemia line offers an attractive alternative. For a recent review see Ann. Rev. Biochem 47: 419-448, 1978. We propose to explore 1) whether MADS DNA is present in Friend's erythroleukemia cells and 2) whether MADS DNA is altered after the cell line is exposed to agents which promote division leading to differentiation to the globin producing state. If both 1 and 2 hold, we can use probes to determine whether MADS DNA is enriched (depleted?) in globin sequences upon stimulation.

We can also pull the globin sequences out of MADS DNA and sequence them in order to determine whether they are normal or scrambled. For instance, Mark Rush (personal communication) has shown that non-chromosomal small polydiperse circular DNA from African green monkey kidney cells contains at least one Alu sequence which is spliced end to end.

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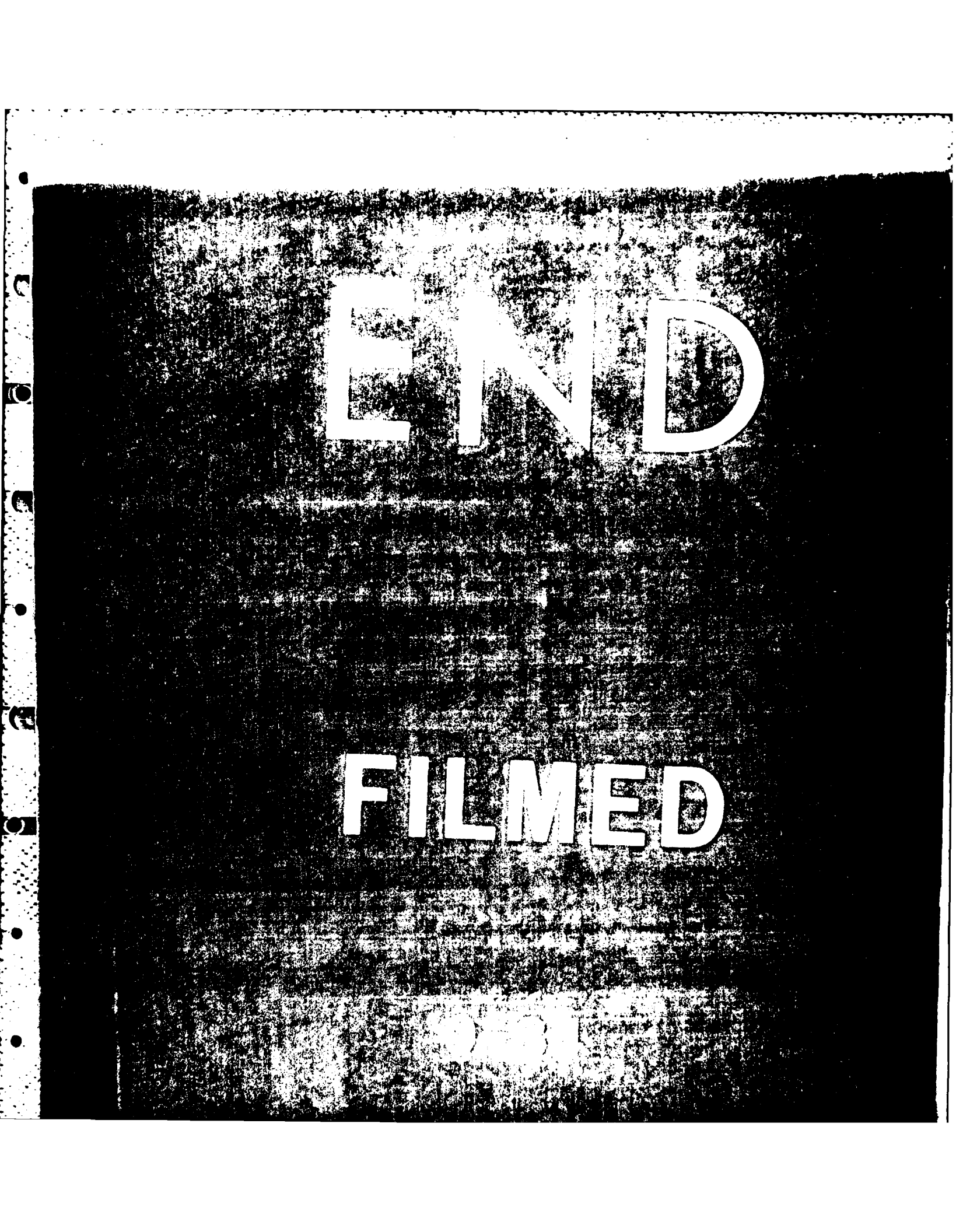
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